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α-2-HS-glycoprotein is a potential marker predicting hepatitis B e antigen seroconversion in patients with chronic hepatitis B during treatment with pegylated interferon alfa-2b

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The efficacy of interferon (IFN) is limited in about 1/3 of patients with chronic hepatitis B (CHB). We used two-dimensional electrophoresis (2-DE)-based proteomic strategies to identify potential serum markers predicting hepatitis B e antigen (HBeAg) seroconversion in these patients during IFN therapy. Two groups of patients were enrolled: training and validation. In the training group, 2-DE experiments and subsequent identification of altered levels of proteins showed that α-2-HS-glycoprotein, leucine-rich α-2-glycoprotein, and haptoglobin were significantly upregulated as compared with baseline levels in the HBeAg seroconversion group, whereas apolipoprotein C-III precursor, leucine-rich α-2-glycoprotein, and α-albumin were downregulated in the non-seroconversion group. For patients with HBeAg seroconversion in the training group, Western blot analyses showed that α-2-HS-glycoprotein levels in 75% of patients were significantly upregulated at the end of the treatment as compared with baseline levels. Subsequent experiments in the validation group showed that α-2-HS-glycoprotein levels were significantly increased at week 4 in 83.33% of patients in the HBeAg seroconversion group. Dynamic changes in the serum level of α-2-HS-glycoprotein may be a potential early marker for predicting HBeAg seroconversion during IFN treatment for CHB.

α-2-HS-glycoprotein, pegylated interferon alfa-2b, chronic hepatitis B

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An estimated 350 million persons worldwide are chronically infected with the hepatitis B virus (HBV) [1]. Chronic infection with HBV can progress to cirrhosis, liver failure, and hepatocellular carcinoma (HCC), and it is a major cause of mortality worldwide [2–4]. Currently, six therapeutic agents have been approved for the treatment of adults with chronic hepatitis B (CHB): interferon (IFN), pegylated IFN alfa (PegIFN alfa), lamivudine, adefovir dipivoxil, entecavir, and L-deoxythymidine [1].

As one of the first-line drugs for the treatment of CHB, IFN has antiviral, antiproliferative, and immunomodulatory effects [5–8]. The advantages of IFN alfa include a finite duration of treatment, a more durable response, and a lack of resistant mutants. The disadvantages of IFN alfa are its cost, side effects, and that its efficacy is limited in about 1/3 of patients [1,9].

In hepatitis B e antigen (HBeAg)-positive patients, the pre-treatment level of alanine aminotransferase (ALT) is a strongest predictor of HBeAg seroconversion during treatments with conventional IFN and PegIFN alfa. Other factors include a high histologic activity index and a low level of HBV DNA. More recently, some studies have suggested that persons infected with HBV genotypes A and B respond better than those infected with genotypes C and D [1,10–12]. In addition to these predictors, other early predictors of

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HBeAg seroconversion during conventional IFN and PegIFN alfa treatments need to be identified.

Proteomics has led to many advances in the understanding of the function and disease of the liver, particularly with regard to HCC [13], liver cirrhosis [14,15], and hepatitis [16,17]. However, proteomic analyses in CHB patients treated with IFN are lacking. As a body fluid with wide clinical uses, the serum has always been an important resource for proteomic analyses. Thus in our study, two-dimensional electrophoresis (2-DE) and mass spectrometry (MS)-based analyses were used to compare the proteomic changes in the serum before and after treatment with IFN alfa-2b for identifying proteins with differentially altered levels. α-2-HS-glycoprotein (also known as "fetuin") was identified as a predictor of seroconversion by Western blot analyses in the training group. Then, α-2-HS-glycoprotein was further validated as a predictor in the validation group. We aimed to identify a potential serum marker for predicting HBeAg seroconversion during IFN treatment in CHB patients.

1 Materials and methods

1.1 Ethical approval of the study protocol

This study was conducted in agreement with the Ethics Committee of Peking University People's Hospital (Beijing, China), and in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from each participant.

1.2 Patients

Two groups of patients were enrolled: training and validation. Patients in the training group met the following criteria: They were adults (18–70 years old); they were positive for hepatitis B surface antigen (HBsAg) for >6 months; they were positive for HBeAg; their HBV DNA levels were >100000 copies mL^{-1}; their serum ALT levels were 2–10 times the upper limit of the normal (ULN) range; they had a white cell count (WBC) $>3.0\times10^9$ L^{-1}; they had a granulocyte count $>1.5\times10^9$ L^{-1}; they had a platelet count $>100\times10^9$ L^{-1}; women of childbearing age had a negative urine pregnancy test. The exclusion criteria for the training group were patients with any causes of liver diseases other than CHB; pregnant and/or breastfeeding women; individuals who had used immune regulators within the previous six months; individuals who had received antiviral therapy within six months before commencement of the study; individuals with decompensated or compensated cirrhosis; subjects with antibodies against human immunodeficiency virus (HIV); subjects with a history of renal dialysis or organ transplantation.

Patients of the validation group met the following criteria: They were adults (18–70 years old); they were positive for HBsAg for >6 months; they were positive for HBeAg; their HBV DNA levels were >20000 IU mL^{-1}; their serum ALT levels were 2–10 times the ULN; they had a granulocyte count $\geqslant1.5\times10^9$ L^{-1}; they had a platelet count $\geqslant80\times10^9$ L^{-1}; and women of childbearing age had a negative urine pregnancy test. The exclusion criteria for the validation group were patients with any causes of liver diseases other than CHB; pregnant and/or breastfeeding women; individuals who had used immune regulators within the previous six months; individuals who had received antiviral therapy within six months before the commencement of the study; individuals with decompensated or compensated cirrhosis; subjects with antibodies against HIV; persons with a history of renal dialysis or organ transplantation.

1.3 Study design

This was a randomized, controlled study. Patients in the training group were treated for 24 weeks and then followed up for 24 weeks. Patients who met the criteria for study entry were randomized to receive 1 μg kg^{-1} PegIFN alfa-2b (12 kD) subcutaneously once weekly (PegIntron, Schering-Plough, Kenilworth, NJ, USA) or 3.0 MIU conventional IFN alfa-2b thrice weekly (IntronA, Schering-Plough). Efficacy was assessed at the end of the 24-week treatment and after 24 weeks of follow-up. Efficacy was indicated by the normalization of ALT levels, suppression of HBV DNA levels to $<10^5$ copies mL^{-1} (PG Biotech, Shenzhen, China), and seroconversion to anti-HBe (Abbott, Wiesbaden, Germany). According to HBeAg and anti-HBe status, these patients were divided into the HBeAg seroconversion group and HBeAg non-seroconversion group. Serum samples were obtained at baseline at the end of 24-week treatment and at the end of 24-week follow-up. Obtained samples were stored at −80°C until analyses.

Patients in the validation group received 1 μg kg^{-1} PegIFN alfa-2b (PegIntron, Schering-Plough) once a week for 24 weeks, 1.5 μg kg^{-1} PegIFN alfa-2b for 24 weeks, or 1.5 μg kg^{-1} PegIFN alfa-2b for 48 weeks. Efficacy was assessed at week 24. The factors indicating efficacy were normalization of ALT levels, suppression of HBV DNA levels to <12 IU mL^{-1} (Cobas Taqman; Roche, Rotkreuz, Germany), and seroconversion to anti-Hbe (MEIA, Abbott, Abbott Park, IL, USA). Serum samples were obtained at baseline, at week 4 and 24. They were stored at −80°C until analyses.

1.4 2-DE of serum samples of the training group

Serum samples obtained at baseline and at the end of the treatment were pooled in the HBeAg seroconversion and non-seroconversion groups. Samples were then subjected to 2-DE analyses as described previously [18]. Briefly, lipids were removed by chloroform extraction. Proteins were pre-

cipitated with cold acetone and dissolved in buffer containing 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mmol L^{-1} ethylenediamine tetra-acetic acid (EDTA), 50 mmol L^{-1} dithiothreitol (DTT), and 1 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF). Total protein concentrations were determined by the Bradford method. Proteins (2 mg) were solubilized in rehydration buffer containing 6 mol L^{-1} urea, 2% CHAPS, 20 mmol L^{-1} DTT, 0.5% immobilized pH gradient (IPG) buffer, and a trace amount of bromophenol blue. Isoelectric focusing (IEF) was carried out with commercially available preformed IPGs (non-linear, pH 3–7, 24 cm; Amersham, Stockholm, Sweden) using an IPGphor IEF System (Amersham). The IPG strips were rehydrated with samples at 40 V for 10 h. Proteins were then focused at 8000 V for 15000 Vh at 20°C. After a three-step equilibration process (two 15-min incubations in DTT buffer followed by a 15-min incubation in iodoacetamide buffer), the IPG strips were positioned on 12% polyacrylamide gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run at a constant power of 5 W/gel for 1 h. The power was switched to 10 W/gel until the front of the bromophenol blue dye reached the bottom of the gel. Gels were then stained with Coomassie Brilliant Blue G-250 and imaged using Labscan (Amersham).

1.5 Image analyses

Digitized images of stained gels were analyzed using ImageMaster 2D software version 5.1 (Amersham). The match analysis was performed in an automatic mode, and the mismatched and unmatched spots were correctly matched by manual editing. The gel with the highest number of spots was selected as the reference gel. The relative volume of each spot was considered to represent its expression level. Only spots that were present in the experimental gels and were altered by at least two-fold as compared to the spots in the control gels were considered to be significant and were subjected to MS for protein identification.

1.6 Digestion and identification of proteins

Spots of interest were excised and digested in the gel with digestion buffer (50 mmol L^{-1} NH_4HCO_3) containing 50 ng of trypsin (sequencing grade; Roche). The digestion buffer containing the digested peptides was vacuum-dried to a final volume of ~10 μL, and the sample stored at –20°C. For liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS-MS), vacuum-dried peptide extracts were dissolved in 0.1% formic acid/2% acetonitrile to a final volume of 20 μL and samples eluted with a 10%–30% gradient of organic phase (0.1% formic acid/acetonitrile) for 30 min. The capillary voltage was 22 V at 200°C, and tandem MS was carried out on precursors with charge states of 2, 3, and 4 covering *m*/*z* from 400 to 1800. Spectra were recorded on an LCQ Deca XP Plus spectrometer (ThermoFinnigan, San Jose, CA, USA). MS/MS spectra were searched for against the National Center for Biotechnology Information (NCBI) NR database using the SEQUEST algorithm. Spectral matches were retained with a minimal cross-correlation score (XCorr) of 1.9, 2.2 and 3.75 for charge states +1, +2 and +3; the delta Cn value was >0.1.

1.7 Western blot analyses

The serum samples of patients with HBeAg seroconversion in the training group and those of patients with or without HBeAg seroconversion in the validation group were homogenized in lysis buffer (50 mmol L^{-1} Tris-Cl (pH 8.0), 1% NP-40, 150 mmol L^{-1} NaCl, 0.1% SDS, 0.02% sodium azide, and 100 μg mL^{-1} PMSF). Serum proteins (50 μg) were resolved by 12% SDS-PAGE and electrotransferred to a nitrocellulose membrane. Membranes were blocked with non-fat dried milk in TBS containing 0.2% Tween-20 (TBST) for 1 h at room temperature. Membranes were then incubated with mouse monoclonal antibody against α-2-HS-glycoprotein (dilution, 1∶1000; Abcam, Cambridge, UK) overnight at 4°C. After washing in TBST thrice, membranes were reacted with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (dilution, 1∶3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Immunodetection was undertaken with an ECL-Plus kit (Pierce Biotechnology, Rockford, IL, USA) in accordance with the manufacturer's instructions.

1.8 Statistical analyses

Data are mean±SD. SPSS version 10.0 (SPSS, Incorporated, Chicago, IL, USA) was used for statistical analyses. Data were analyzed using the paired samples *t*-test. $P<0.05$ was considered significant.

2 Results

2.1 Patient characteristics

Twenty-nine patients (22 males) were enrolled in the training group. The mean age (±SD) was 32.38±10.53 years. Of these 29 patients, 15 patients were randomized to receive 1 μg kg^{-1} PegIFN alfa-2b once weekly and 14 patients were randomized to receive 3 MIU of IFN alfa-2b thrice weekly for 24 weeks. All patients completed the 24 weeks of treatment. One patient was lost to follow-up.

Twenty-nine patients (24 males) were enrolled in the validation group. The mean age (±SD) was 28.59±8.94 years. Of these 29 patients, 10 patients received 1 μg kg^{-1} PegIFN alfa-2b for 24 weeks, nine patients received 1.5 μg

kg^{-1} PegIFN alfa-2b for 24 weeks, and 10 patients received 1.5 μg kg^{-1} PegIFN alfa-2b for 48 weeks once weekly. All patients completed the 24 weeks of treatment. Baseline demographic and other characteristics of the training group and validation group are shown in Table 1. Baseline characteristics of patients with/without HBeAg seroconversion in the training group and validation group are listed in Table 2.

Table 1 Baseline characteristics of the training and validation groups

Characteristic	Training group (*n*=29)	Validation group (*n*=29)
Males, number (%)	22(75.86)	24(82.76)
Race, number (%)		
Asian	100	100
Age, years (mean±SD)	32.38±10.53	28.59±8.94
ALT, U L^{-1}		
Mean±SD	180.21±94.91	170.72±80.53
Median	152.00	156.00
Range	82–384	82–390
HBV DNA-log[a)]		
Mean±SD	8.24±0.83	7.61±1.27
Median	8.18	7.98
Range	6.70–9.93	4.35–9.03

a) HBV DNA of the training group, copies mL^{-1}; HBV DNA of validation group, IU mL^{-1}.

2.2 Efficacy of IFN therapy in CHB patients

At the end of the 24-week treatment, ALT levels were normal in nine patients in the training group, and HBV DNA levels decreased to <100000 copies mL^{-1} in six patients. However, HBeAg seroconversion did not occur in any patients. One patient was lost to follow-up, so 28 patients completed the 24-week follow-up. Of these, six patients had normalized ALT levels and five patients had HBV DNA levels <100000 copies mL^{-1}; HBeAg seroconversion was observed in four patients. Table 3 lists the clinical data of patients with HBeAg seroconversion at baseline and at the end of follow-up.

In the validation group, ALT levels were normal in 10 patients at week 24, HBV DNA levels did not decrease to <12 IU mL^{-1} in any of the patients, but HBeAg seroconversion occurred in six patients. The clinical data of patients with and without HBeAg seroconversion at baseline and at week 24 are shown in Table 4.

2.3 Significantly altered levels of serum proteins in patients treated with IFN

At the end of the 24-week treatment, HBeAg seroconversion was not observed in any patient in the training group. However, at the end of follow-up, HBeAg seroconversion was observed in four patients. Thus, the serum samples obtained from these four patients at baseline were pooled, as were the samples obtained at the end of the 24-week treatment. Similarly, the serum samples obtained at baseline from the 24 patients without HBeAg seroconversion were pooled, as were those obtained at the end of the 24-week treatment. Proteins in the serum of the patients in the two groups were significantly altered. Proteins with differentially altered levels were then identified by searching MS/MS spectra against the protein database. Compared with baseline, 21 protein spots were significantly altered at the end of the 24-week treatment in the HBeAg seroconversion group, and 10 protein spots were significantly altered at the end of the 24-week treatment in HBeAg non-seroconversion group. However, some protein spots were subsequently found to have overlapping identities according to MS/MS spectra. Thus, compared with baseline, α-2-HS-glycoprotein, leucine-rich α-2-glycoprotein, and haptoglobin were upregulated in the HBeAg seroconversion group. Moreover, apolipoprotein C-III precursor, leucine-rich α-2-glycoprotein, and α-albumin were downregulated in the HBeAg non-seroconversion group. The results of 2-DE analyses of the serum proteins are shown in Figure 1 and Table 5.

Table 2 Baseline characteristics of patients with/without HBeAg seroconversion in the training group and validation group

Training group			
	HBeAg seroconversion	HBeAg non-seroconversion	*P*
Age (mean±SD)	22±2.71	34±10.37	0.031
Male:female ratio	3∶1	19∶6	0.965
ALT, mean±SD	111.5±34.28	191.2±97.22	0.121
HBV DNA[a)], median (range)	6.6×10^7 (6.23×10^6–1.6×10^8)	1.9×10^8 (5.0×10^6–8.6×10^9)	0.114
Genotype (B∶C)	3∶1	2∶23	0.001
Validation group			
	HBeAg seroconversion	HBeAg non-seroconversion	*P*
Age (mean±SD)	24.50±4.46	29.65±9.57	0.215
Male:female ratio	5∶1	19∶4	0.967
ALT, mean±SD	210.33±100.38	160.39±73.65	0.181
HBV DNA[a)], median (range)	1.1×10^7 (2.2×10^4–1.3×10^8)	1.2×10^8 (5.84×10^4–1.1×10^9)	0.018
Genotype (B∶C)	2∶4	11∶12	0.525

a) HBV DNA of the training group, copies mL^{-1}; HBV DNA of validation group, IU mL^{-1}.

Table 3 Clinical data of patients with HBeAg seroconversion at the baseline and at the end of follow-up in the training group

Patient No.	Sex	Age	Baseline					End of follow-up				
			ALT	AST	HBV DNA	HBeAg	Anti-HBe	ALT (U L^{-1})	AST (U L^{-1})	HBV DNA (copies mL^{-1})	HBeAg	Anti-HBe
1	M	23	100	64	1.64×10^8	+	–	29	31	1.34×10^4	–	+
2	M	18	161	65	1.22×10^8	+	–	19	25	2.69×10^3	–	+
3	F	23	103	93	9.7×10^6	+	–	14	26	2.44×10^3	–	+
4	M	24	82	50	6.23×10^6	+	–	41	35	<1000	–	+

Table 4 Clinical data of patients with and without HBeAg seroconversion at the baseline and at the end of treatment in the validation group

Patient No.	Sex	Age	Baseline					End of treatment				
			ALT	AST	HBV DNA (IU mL^{-1})	HBeAg	Anti-HBe	ALT (U L^{-1})	AST (U L^{-1})	HBV DNA (IU mL^{-1})	HBeAg	Anti-HBe
1	M	24	238	122	2.22×10^4	+	–	62	52	345	–	+
2	F	23	157	104	5.6×10^7	+	–	15	25	1.17×10^4	–	+
3	M	21	380	269	1.25×10^8	+	–	14	20	976	–	+
4	M	21	248	85	1.5×10^7	+	–	52	37	219	–	+
5	M	25	125	46	2.65×10^5	+	–	77	46	2270	–	+
6	M	33	114	86	7.71×10^6	+	–	41	42	3950	–	+
7	M	35	88	34	9.29×10^7	+	–	13	20	3.26×10^5	+	–
8	M	39	121	56	5.84×10^4	+	–	15	16	4600	+	–
9	M	26	134	54	4.48×10^8	+	–	144	66	1.53×10^8	+	–
10	M	45	116	58	1.03×10^5	+	–	143	80	2.11×10^5	+	–
11	M	22	133	56	6.26×10^7	+	–	90	58	1.38×10^7	+	–
12	M	22	97	45	3.36×10^6	+	–	26	22	2.65×10^5	+	–
13	M	26	191	107	6.47×10^8	+	–	188	90	2.96×10^8	+	–
14	M	31	198	103	2.12×10^8	+	–	25	22	3.25×10^7	+	–
15	M	18	180	80	1.07×10^8	+	–	160	93	9.11×10^7	+	–
16	F	57	100	41	9.6×10^8	+	–	186	194	3.23×10^7	+	–
17	M	30	92	51	4.02×10^7	+	–	42	27	2.09×10^5	+	–
18	M	23	149	72	9.1×10^7	+	–	138	66	1.04×10^7	+	–
19	M	43	288	284	1.5×10^8	+	–	42	32	7.68×10^7	+	–
20	M	27	268	108	1.06×10^9	+	–	595	274	1.03×10^7	+	–
21	F	20	174	86	4.32×10^7	+	–	45	28	3.74×10^6	+	–
22	M	25	170	36	1.22×10^8	+	–	76	42	1.42×10^7	+	–
23	M	28	160	36	1.22×10^7	+	–	57	35	2.27×10^7	+	–
24	F	31	156	30	6.34×10^8	+	–	26	27	8.1×10^7	+	–
25	F	37	390	177	3.7×10^8	+	–	45	37	7390	+	+
26	M	19	136	34	1.38×10^8	+	–	26	25	3.89×10^7	+	–
27	M	24	82	40	3.14×10^8	+	–	31	31	9.68×10^7	+	–
28	M	33	182	106	3.17×10^8	+	–	43	24	1.1×10^7	+	–
29	M	21	84	44	9.64×10^7	+	–	18	27	2.88×10^7	+	–

2.4 Upregulated expression of α-2-HS-glycoprotein at the end of treatment as a potential predictor of HBeAg seroconversion at the end of follow-up in the HBeAg seroconversion group

In the training group, the serum samples obtained at the baseline and at the end of the 24-week treatment in the HBeAg seroconversion group were subjected to Western blot analyses. α-2-HS-glycoprotein levels in 75% of patients (3/4) were significantly upregulated at the end of the 24-week treatment as compared with baseline levels (all $P<0.05$). α-2-HS-glycoprotein levels in 25% of patients (1/4) showed no significant differences ($t=3.166$, $P=0.087$) (Figure 2). The results suggested that, at the end of the 24-week treatment, if α-2-HS-glycoprotein levels in serum are upregulated as compared with baseline levels, HBeAg seroconversion may probably occur in these patients at the end of follow-up. Thus, α-2-HS-glycoprotein may be regarded as a predictor of HBeAg seroconversion during IFN treatment.

2.5 Increased serum level of α-2-HS-glycoprotein at week 4 as a potential early predictor of HBeAg seroconversion during PegIFN treatment

Further confirmation of our results was carried out in the validation group. α-2-HS-glycoprotein levels in serial serum samples were evaluated by Western blot analyses. In the HBeAg seroconversion group (six patients), α-2-HS-glycoprotein levels in 83.33% of patients (5/6) were significantly upregulated at week 4 as compared with baseline levels ($P<0.05$); α-2-HS-glycoprotein levels in 16.67% of

Table 5 Differentially altered serum levels of proteins at the end of treatment compared with baseline in pooled sera of patients in the HBeAg seroconversion group and HBeAg non-seroconversion group

Group	Spot No.	Matched peptides	Name	Fold change	GenBank accession ID.	pI/M_r	Biological function
HBeAg seroconversion	1272	LDGKFSVVYAK AQLVPLPPSTYVEFTVSGTDCVAK EATEAAKCNLLAEK TEAAKCNLLAEK HTFMGVVSLGSPSGEVSHPR GPLQLER	α-2-HS-glycoprotein	↑ 2.72±0.34	gi|4502005	5.43/39000	Receptor signaling protein tyrosine kinase inhibitor activity; protein binding
	1350	ALGHLDLSGNR VAAGAFQGLR LHLEGNKLQVLGK CAGPEAVK DLLLPQPDLR	Leucine-rich α-2-glycoprotein	↑ 2.66±0.17	gi|16418467	6.45/38000	Protein binding; molecular function
	1404	TEGDGVYTLNDKK TEGDGVYTLNNEK LPECEAVCGK VMPICLPSK	Haptoglobin	↑ 2.34±0.11	gi|4826762	6.13/45000	Catalytic activity; hemoglobin binding
HBeAg non-seroconversion	384 & 387	GWVTDGFSSLK	Apolipoprotein C-III precursor	↓ 4.53±0.41	gi|4557323	5.23/11000	Cholesterol binding; lipid transporter activity
	244	ALGHLDLSGNR VAAGAFQGLR CAGPEAVK DMVEYKDR	Leucine-rich α-2-glycoprotein	↓ 2.99±0.32	gi|16418467	6.45/38000	Protein binding; molecular function
	39	ESLLNHFLYEVAR VVHFIYIAILSQK FTDSENVCQER HPDLSIPELLR MVQQECKHFQNLGK	α-albumin	↓ 2.67±0.19	gi|4501987	5.64/69000	Protein binding; toxin binding

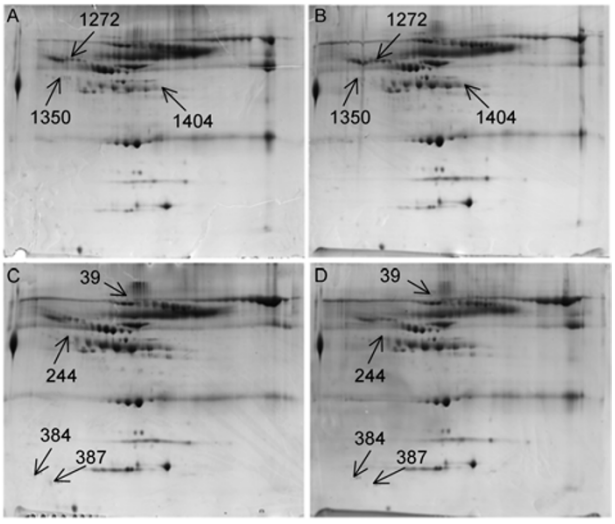

Figure 1 Two-dimensional electrophoresis of serum proteins. A, HBeAg seroconversion group at baseline; B, HBeAg seroconversion group at the end of treatment; C, HBeAg non-seroconversion group at baseline; D, HBeAg non-seroconversion group at the end of treatment. In A and B, 1272 refers to α-2-HS-glycoprotein, 1350 refers to leucine-rich α-2-glycoprotein, and 1404 refers to haptoglobin. In C and D, 384 and 387 refer to apolipoprotein C-III precursor, 244 refers to leucine-rich α-2-glycoprotein, and 39 refers to α-albumin.

patients (1/6) showed no significant differences (t=0.467, P=0.687) (Figure 3). However, in the HBeAg non-seroconversion group (23 patients), α-2-HS-glycoprotein levels in 52.17% of patients (12/23) were significantly downregulated at week 4 as compared with baseline levels (P<0.05); levels in 47.83% (11/23) of patients showed no significant differences (P>0.05) (Figure 4).

These results suggested that, if α-2-HS-glycoprotein levels in serum were significantly upregulated at week 4 as compared with baseline levels, HBeAg seroconversion would probably occur at week 24. Thus, upregulated expression of α-2-HS-glycoprotein at week 4 may be regarded as an early predictor of HBeAg seroconversion during PegIFN treatment.

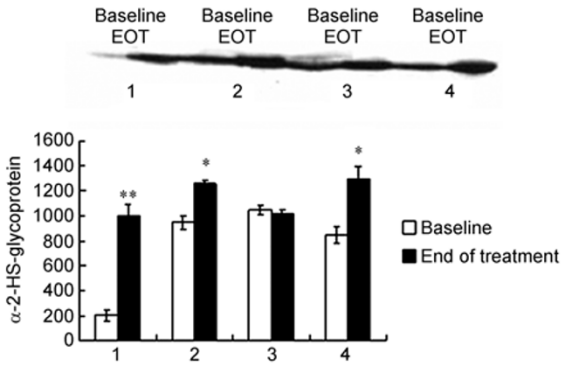

Figure 2 Dynamic changes in α-2-HS-glycoprotein levels in patients with HBeAg seroconversion in the training group. Nos. 1–4 refer to the first to fourth patient, and EOT refers to the end of treatment. The Y axis represents the volume of bands analyzed using Quantity One software. α-2-HS-glycoprotein levels in 75% of patients were upregulated at the end of treatment as compared with baseline levels. *, P<0.05; **, P<0.01.

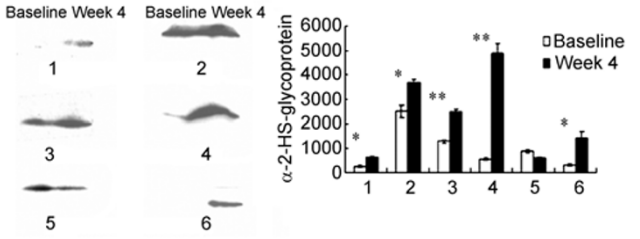

Figure 3 Dynamic changes in the α-2-HS-glycoprotein levels of patients with HBeAg seroconversion in the validation group. Nos. 1 to 6 refer to the first to sixth patient. The *Y* axis represents the volume of the bands analyzed using Quantity One software. α-2-HS-glycoprotein levels in 83.33% of the patients were significantly upregulated at week 4 as compared with baseline levels.*, $P<0.05$; **, $P<0.01$.

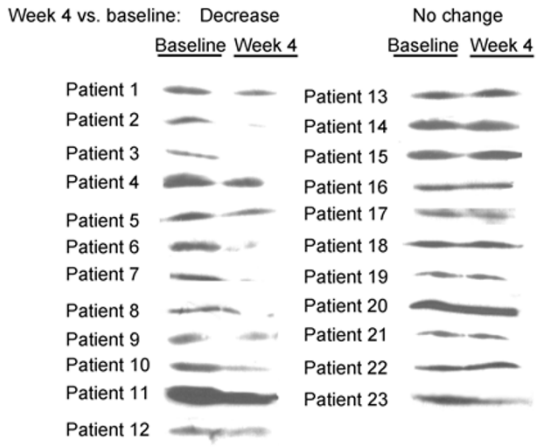

Figure 4 Dynamic changes in the α-2-HS-glycoprotein levels of patients without HBeAg seroconversion in the validation group. Comparing week 4 with baseline, the serum level of α-2-HS-glycoprotein decreased in 12 patients and did not change in 11 patients. No significant increases were observed in any patient.

3 Discussion

The proteomic approach is considered to be the key technology in the global analyses of protein expression [19]. The 2-DE method, together with MS, has greatly promoted systematic analyses of global expression of protein, which provides new insights into the pathogenetic mechanisms of various human diseases. Such improved capability to identify disease-specific profiles of proteins leads to more accurate prediction of disease class and development of novel treatments [20].

HBV infection is a serious problem worldwide; >350 million people have chronic infections. CHB is the 10th leading cause of death worldwide. Two types of therapies are currently available for CHB: IFN-alfa therapy and therapy with nucleosides or nucleotide analogs (e.g., lamivudine, adefovir dipivoxil). IFN alfa is a recombinant IFN with antiviral, antitumor, and immunomodulatory properties [5–8]. Seroconversion from HBeAg to anti-HBe is usually associated with a favorable outcome and sustained biochemical and histologic remission [21–23], so it is regarded as a major indicator of efficacy during antiviral treatment. Proteomic analyses in CHB patients treated with IFN are lacking. We used 2-DE technology to analyze serum samples in CHB patients treated with IFN for identifying proteins with differentially altered levels after IFN therapy.

Six proteins with differentially altered levels were identified in the present study. Levels of α-2-HS-glycoprotein, leucine-rich α-2-glycoprotein, and haptoglobin were upregulated as compared with baseline levels in the HBeAg seroconversion group. Expression of apolipoprotein C-III precursor, leucine-rich α-2-glycoprotein, and α-albumin was downregulated in the non-seroconversion group. α-2-HS-glycoprotein is an abundant plasma protein synthesized predominantly in the liver; it is a natural inhibitor of the insulin receptor tyrosine kinase [24,25]. It is also a transforming growth factor-β type-II receptor mimic and cytokine antagonist [26]. Phosphofetuin synthesized by hepatocytes may be a natural modulator of hepatocyte growth factor as a chalone, and regulation of expression of phosphofetuin by growth factors and cytokines may be involved in liver regeneration under inflammatory conditions such as hepatitis [27].

In 2002, Kalabay *et al.* [28] found significantly lower levels of α-2-HS-glycoprotein in patients with liver cirrhosis and HCC. A strong positive correlation was found with serum transferrin, albumin, and prothrombin. Of all laboratory parameters studied, serum α-2-HS-glycoprotein levels showed the greatest difference between patients who died of cirrhosis or cancer and patients who survived. A multiple logistic regression analysis indicated that the decrease in serum levels of α-2-HS-glycoprotein was independent of all other variables that were found to be decreased in deceased patients. Thus, a decreased serum concentration of α-2-HS-glycoprotein was hypothesized to be caused by hepatocellular dysfunction rather than an acute-phase reaction, and was postulated to be an outstanding predictor of short-term mortality in patients with liver cirrhosis and liver cancer [28]. In 2007, Kalabay *et al.* [29] found that decreased serum levels of α-2-HS-glycoprotein were associated with long-term mortality. They found that 41 patients died during the 12-month follow-up period and that the deceased patients had lower baseline levels of α-2-HS-glycoprotein than the 52 patients who survived. Of all the laboratory parameters evaluated, the serum level of α-2-HS-glycoprotein, the Child-Pugh score and Model for end-stage liver disease (MELD) score showed the greatest difference between deceased patients and patients who survived. A multivariate analysis revealed that the correlation of low serum levels of α-2-HS-glycoprotein to mortality was stronger than the correlations of Child-Pugh score and MELD score to mortality [29].

α-2-HS-glycoprotein is an abundant plasma protein syn-

thesized predominantly in the liver. Hence, regulation of expression of phosphofetuin by growth factors and cytokines may be involved in liver regeneration under inflammatory conditions such as hepatitis. Thus, α-2-HS-glycoprotein is an outstanding predictor of short-term mortality in patients with liver cirrhosis and liver cancer. The correlation of low serum levels of α-2-HS-glycoprotein to mortality is stronger than the correlation of CP and MELD scores to mortality, and the present study revealed that α-2-HS-glycoprotein levels were upregulated as compared with baseline levels in the HBeAg seroconversion group. We therefore chose α-2-HS-glycoprotein for further validation by Western blot analyses.

The results of the Western blot analyses showed that, in the HBeAg seroconversion group, serum levels of α-2-HS-glycoprotein in 75% of patients were significantly upregulated at the end of treatment as compared with baseline levels, and that the levels in 25% of patients showed no significant differences. These results suggested that α-2-HS-glycoprotein may be regarded as a predictor of HBeAg seroconversion during IFN treatment.

The advantages of IFN alfa include a finite duration of treatment, a more durable response, and a lack of resistant mutants. However, the efficacy of IFN alfa is limited in about 1/3 of patients, the adverse effects of IFN are well documented, and IFN is very expensive. Studies have shown that, in HBeAg-positive patients, the pretreatment ALT level is the strongest predictor of HBeAg seroconversion during treatment with conventional IFN and PegIFN alfa. Other factors include a high histologic activity index, low level of HBV DNA, and genotypes [1,5–7]. In addition to these predictors, other predictors for HBeAg seroconversion in IFN therapy are urgently needed.

In the training group, we found that α-2-HS-glycoprotein may be regarded as a predictor of HBeAg seroconversion in IFN treatment. Thus, to further validate α-2-HS-glycoprotein as a predictor, dynamic changes in the serum level of α-2-HS-glycoprotein were detected in the validation group by Western blot analyses. In the HBeAg seroconversion group, α-2-HS-glycoprotein levels in 83.33% of patients were upregulated at week 4 as compared with baseline levels. Simultaneously, in the HBeAg non-seroconversion group, α-2-HS-glycoprotein levels in 52.17% of patients were downregulated at week 4 as compared with baseline levels, and levels in 47.83% patients showed no significant differences.

These results suggested that, at week 4, if the α-2-HS-glycoprotein level in the serum of a patient is significantly upregulated, HBeAg seroconversion might occur in that patient at week 24. Conversely, if the serum α-2-HS-glycoprotein level is not markedly upregulated or is downregulated, HBeAg seroconversion will probably not occur at week 24.

4 Conclusion

The levels of six serum proteins were shown to be differentially altered after IFN therapy in CHB patients. Further study showed that expression of α-2-HS-glycoprotein was upregulated in the HBeAg seroconversion group at week 4 when compared with baseline levels. This suggested that dynamic changes in the serum level of α-2-HS-glycoprotein may be a potential early marker predicting HBeAg seroconversion in IFN treatment for CHB. Considering the limited number of patients in the present study, further work will be carried out to explore the applicability of this marker to clinical practice.

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